Perspective

Investigating the role of Ca^{2+}/calmodulin-dependent protein kinase II in the survival of retinal ganglion cells

Xinzheng Guo, Bo Chen[*]

Retinal ganglion cells (RGCs) are the sole output neurons of the retina that project long axons and transmit visual information to the brain. The degeneration of RGCs leads to irreversible vision loss in a variety of pathological states, including excitotoxicity, traumatic nerve injury, and glaucoma. However, an unmet clinical challenge is the lack of effective neuroprotective approaches to protect RGCs and thus preserve their function, necessitating extensive investigation of pro-survival genes in basic and translational research. Here, we first briefly describe widely used experimental models of RGC degeneration and methods for evaluating gene function in RGC survival, and then share our thoughts on the role of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in regulating the survival of RGCs after excitotoxic injury.

Experimental models of RGC degeneration: (1) Excitotoxicity model: Historically, excitotoxicity was first demonstrated to kill the inner retinal neurons after systematic glutamate injections. In adult mice, a single injection of high dose of N-methyl-D-aspartate (NMDA) into the vitreous chamber will induce massive RGC death (Nakamura et al., 2021) (**Figure 1A**). In our hands, an intravitreal administration of toxic levels of NMDA solution (1.5 μL, 20 mM) will lead to more than 80% of RGC loss one week after injection in the adult mouse retina. (2) Optic nerve crush model: The optic nerve in mice can be easily accessed intraorbitally and crushed by fine forceps. Optic nerve crush is a widely used model to investigate RGC survival and axon regeneration (**Figure 1B**). Axotomy caused by optic nerve crush results in delayed death of RGCs, with about 75% loss of RGCs 2 weeks after injury (Guo et al., 2016). (3) Glaucoma model: Several induced and genetic models have been developed to mimic elevated intraocular pressure (IOP) of glaucoma patients (**Figure 1C**). Injection of microbeads into the anterior chamber to occlude aqueous outflow is a popular way to induce ocular hypertension in mice (Calkins et al., 2018). The RGC loss is about 30% at 8 weeks after microbead injection (Yang et al., 2016). (4) Ischemia/reperfusion model: Retinal ischemia/reperfusion could be induced by raising the IOP to ultra-high level (such as 120 mm Hg) for 60 minutes by placing a needle connecting an elevated saline reservoir to the anterior chamber (**Figure 1D**). Ischemia results in a significant loss of RGCs. In the meantime, ischemia also leads to loss of other inner retinal neurons.

Methods to evaluate gene function in RGC survival: To evaluate the role of a gene in RGC survival, initial examination often involves probing gene expression levels or its enzymatic activities before and after injury. Mechanistic investigation requires gain-of-function and loss-of-function assays. Although chemical activators and inhibitors can be used to alter gene function or enzymatic activities, more convincing results require direct manipulation of gene expression or enzymatic activities. Intravitreal injection of AAVs (adeno-associated viruses) is an efficient way to deliver genes into RGCs (**Figure 1E**). When combined with molecular and genetic techniques such as site-directed mutagenesis, Cre-LoxP, and CRISPR-Cas9, AAV-mediated gene transfer could achieve various purposes to manipulate a gene.

Quantification of the RGC numbers from the gain-of-function and loss-of-function studies of a gene will help determine its role in RGC survival.

Clear and definitive labeling of RGCs is a prerequisite for RGC quantification. The well-established method to label all RGCs is to perform immunohistochemistry using antibodies recognizing neuron-specific class III beta-tubulin (Tuj1) (Guo et al., 2016) or RNA-binding protein with multiple splicing (RBPMS) (Masin et al., 2021). Tuj1 and RBPMS are more reliable markers for RGC quantification compared to others such as Brn3a/3b as their expression levels may have been downregulated at the early phase of RGC degeneration after injury onset.

RGC density in mice varies in different retinal quadrants as well as different distances from the retinal center. To achieve objective comparison of RGC numbers among experimental and control groups, sampling multiple regions in retinal flat-mounts from all quadrants at a fixed radius is a more reliable method than sampling retinal sections. Based on our experience, following Tuj1 immunostaining, sampling square regions located ~500 μm from the edge of retinal flat-mounts provides consistent quantification of RGC numbers as these regions contain less dense RGC axons allowing clearly discernible RGC somas to be scored (Guo et al., 2016) (**Figure 1F**).
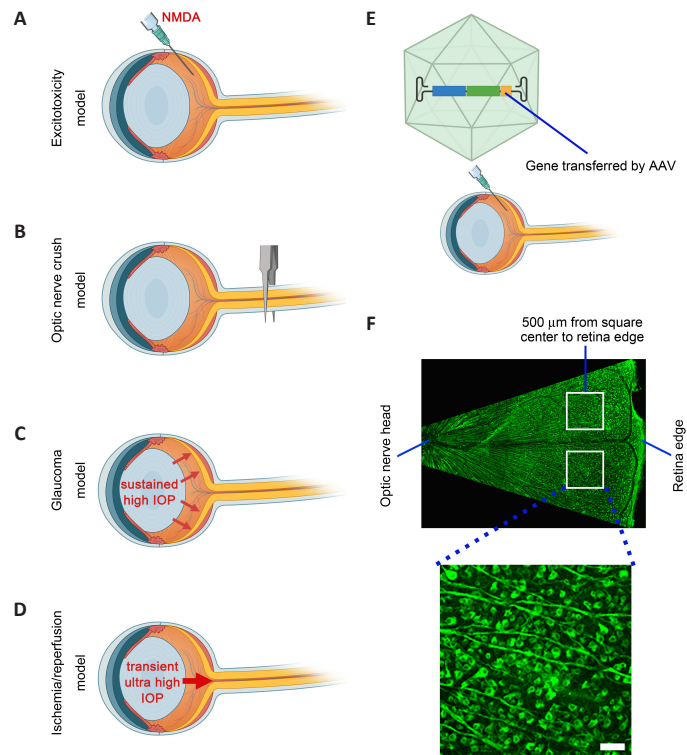

Figure 1 │ Experimental models of RGC degeneration and methods to evaluate gene function in RGC survival.
(A) Excitotoxicity model via intravitreal injection of NMDA. (B) Optic nerve crush model via intraorbital crush with a pair of forceps. (C) Glaucoma model with sustained high IOP. (D) Ischemia/reperfusion model with transient ultra-high IOP. (E) AAV-mediated gene delivery into RGCs via intravitreal injection. (F) Tuj1 immunohistochemistry in the flat-mounted mouse retina, and sampling square regions for RGC quantification. Unpublished data. Scale bar: 40 μm. AAV: Adeno-associated virus; IOP: intraocular pressure; NMDA: N-methyl-D-aspartate. Created with BioRender.com.

Perspective

CaMKII basics: Calcium (Ca^{2+}) is an essential messenger that regulates numerous cellular activities. Aberrant Ca^{2+} dynamics, taking place in RGCs after excitotoxic insults and optic nerve injury, often leads to cell death. CaMKII, as a central coordinator and executor of Ca^{2+} signal transduction, regulates a broad array of cellular functions, including homeostatic balance of the cell and activity-dependent neuronal plasticity (Bayer and Schulman, 2019). CaMKII consists of a group of isoforms derived from four closely related genes (α, β, γ, and δ). CaMKIIα and CaMKIIβ are the two major isoforms highly expressed in the central nervous system including retinal ganglion cells. Thus, CaMKII may play an important role in regulating RGC survival. The structure and regulatory mechanism of CaMKII is now well understood. Each CaMKII isoform contains a catalytic, an autoinhibitory, and an association domain. The catalytic domain, containing the ATP- and substrate-binding sites, is capable of catalyzing the phosphotransferase reaction. Ca^{2+}/calmodulin binding activates CaMKII by opening the autoregulatory domain and allowing autophosphorylation at a critical site to generate Ca^{2+}/calmodulin-independent autonomous activity (Bayer and Schulman, 2019). Over many years of mechanistic studies, the gain-of-function and loss-of-function mutants of CaMKII have been characterized and widely used in neuroscience research (Bayer and Schulman, 2019). Using AAV-mediated gene delivery of various CaMKII mutants into RGCs, the role of CaMKII in regulating RGC survival could be thoroughly investigated in experimental models of RGC degeneration.

Current understanding of CaMKII in RGC survival: Studies of CaMKII in RGCs are surprisingly limited, and consequently, the role of CaMKII in regulating RGC survival is poorly understood in physiological and pathophysiological conditions. So far, the only studies of CaMKII in RGC survival are limited to the excitotoxicity model. Therefore, our discussion is focused on the role of CaMKII in the excitotoxic death of RGCs.

Using *in vitro* biochemical assays of the whole retinal homogenates, CaMKII activity was found to be elevated for short periods of time and then decreased after intravitreal injection of NMDA (Laabich and Cooper, 2000; Takeda et al., 2007). Interestingly, co-treatment of the CaMKII inhibitor AIP (myristoylated autocamtide-2-related inhibitory peptide) with NMDA partially protected RGCs from excitotoxic cell death (Laabich and Cooper, 2000; Goebel, 2009). However, siRNA-mediated CaMKII knockdown significantly accelerated NMDA-induced RGC loss in another study (Takeda et al., 2007). These seemingly contradictory results are reminiscent of those obtained from cortical neuron cultures, where CaMKII inhibition seems to be neuroprotective immediately after the excitotoxic insult, however, it induces neuronal death in a long period of time (Ashpole and Hudmon, 2011).

What exactly is the role of CaMKII in RGC survival? To reveal the role of CaMKII in RGC survival, it would be more definitive to examine and manipulate CaMKII specifically in RGCs. For example, the initial re-examination could focus on determining whether CaMKII expression levels or activities are up-regulated or down-regulated specifically in RGCs after NMDA-induced excitotoxicity. Studies using whole retinal homogenates for immunoblotting or CaMKII kinase assays may not reflect the changes taking place in RGCs because CaMKII is also highly expressed in other retinal neurons such as amacrine cells. Similarly, although AIP is effective to inhibit CaMKII activity, it affects all retinal cell types when administered intravitreally. Therefore, the previously reported protective effects of AIP treatment may not directly result from inhibition of CaMKII in RGCs. The precise role of CaMKII in regulating RGC survival after excitotoxic insults remains to be unraveled.

In physiological conditions, Ca^{2+} influx into neurons through NMDA receptors will activate CaMKII to initiate downstream signal transduction. The key question is: do excitotoxic levels of NMDA inevitably activate CaMKII in RGCs? In cultured cortical neurons, low concentrations of glutamate or NMDA increased CaMKII activity whereas high concentrations of glutamate or NMDA reduced CaMKII activity (Zhou et al., 2012). It will be very interesting to examine how CaMKII in RGCs responds to low and high concentrations of glutamate or NMDA.

Conclusion and prospective: CaMKII is an essential regulator of cellular homeostasis and neuronal activities. It may play an important role in regulating the survival of RGCs and thus preserving visual function in normal and diseased retinas. Current studies have not yet revealed the precise role of CaMKII in protecting RGCs from excitotoxic cell death. Definitive studies are required to comprehensively and thoroughly investigate this unsolved question. CaMKII assays specifically for RGCs such as detecting CaMKII levels/activities from purified RGCs or immunohistochemistry using antibodies recognizing RGCs and the active form of CaMKII will help answer how CaMKII activities change in RGCs after excitotoxic injury. Furthermore, modulation of CaMKII activity by AAV-mediated gene delivery of constitutively active or kinase dead mutants of CaMKII will help answer how CaMKII may protect RGCs from excitotoxic cell death. Once we know more about what CaMKII does in RGCs after excitotoxic injury, future studies can be expanded to examine the role of CaMKII in other RGC disease/injury models, such as optic nerve crush and glaucoma models. It remains to be seen whether CaMKII can be used as a general therapeutic target for protection of RGCs and preservation of vision.

Xinzheng Guo, Bo Chen[*]
Departments of Ophthalmology and Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY, USA

*Correspondence to: Bo Chen, PhD, bo.chen@mssm.edu.
https://orcid.org/0000-0003-2768-9007 (Bo Chen)

References

Ashpole NM, Hudmon A (2011) Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. Mol Cell Neurosci 46:720-730.

Bayer KU, Schulman H (2019) CaM kinase: still inspiring at 40. Neuron 103:380-394.

Calkins DJ, Lambert WS, Formichella CR, McLaughlin WM, Sappington RM (2018) The microbead occlusion model of ocular hypertension in mice. Methods Mol Biol 1695:23-39.

Goebel DJ (2009) Selective blockade of CaMKII-alpha inhibits NMDA-induced caspase-3-dependent cell death but does not arrest PARP-1 activation or loss of plasma membrane selectivity in rat retinal neurons. Brain Res 1256:190-204.

Guo X, Snider WD, Chen B (2016) GSK3beta regulates AKT-induced central nervous system axon regeneration via an eIF2Bepsilon-dependent, mTORC1-independent pathway. Elife 5:e11903.

Laabich A, Cooper NG (2000) Neuroprotective effect of AIP on N-methyl-D-aspartate-induced cell death in retinal neurons. Brain Res Mol Brain Res 85:32-40.

Masin L, Claes M, Bergmans S, Cools L, Andries L, Davis BM, Moons L, De Groef L (2021) A novel retinal ganglion cell quantification tool based on deep learning. Sci Rep 11:702.

Nakamura N, Honjo M, Yamagishi R, Kurano M, Yatomi Y, Watanabe S, Aihara M (2021) Neuroprotective role of sphingolipid rheostat in excitotoxic retinal ganglion cell death. Exp Eye Res 208:108623.

Takeda H, Kitaoka Y, Hayashi Y, Kumai T, Munemasa Y, Fujino H, Kobayashi S, Ueno S (2007) Calcium/calmodulin-dependent protein kinase II regulates the phosphorylation of CREB in NMDA-induced retinal neurotoxicity. Brain Res 1184:306-315.

Yang L, Li S, Miao L, Huang H, Liang F, Teng X, Xu L, Wang Q, Xiao W, Ridder WH, 3rd, Ferguson TA, Chen DF, Kaufman RJ, Hu Y (2016) Rescue of glaucomatous neurodegeneration by differentially modulating neuronal endoplasmic reticulum stress molecules. J Neurosci 36:5891-5903.

Zhou X, Zheng F, Moon C, Schluter OM, Wang H (2012) Bi-directional regulation of CaMKIIalpha phosphorylation at Thr286 by NMDA receptors in cultured cortical neurons. J Neurochem 122:295-307.

C-Editors: Zhao M, Zhao LJ, Qiu Y; T-Editor: Jia Y